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Analysis and Quantitative Determination of Group B Saponins in Processed Soybean Products†‡

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Pure analytical standards for the major saponins present in processed soy products, the group B saponins (soyasaponins I, II, III and IV), were isolated in mg quantities by a combination of processing, precipitation/re-solubilisation, TLC and preparative HPLC. These standards were determined to be pure by LC-ESI/MS analysis and NMR. The standards were used to perfect a facile analytical HPLC method using spectrometric detection to determine the percent composition of the group B soyasaponins in various products from processing of soybean. Published in 2002 by John Wiley & Sons, Ltd.

Keywords: High-performance liquid chromatography; mass spectrometry; quantitative determination; saponin; soybean; *Glycine max*.

INTRODUCTION

Saponins are naturally occurring triterpenoids found in many food materials derived from plants. They are secondary plant metabolites containing a steroid or triterpenoid aglycone with a number of different carbohydrate moieties which are linked through either an ether or an ester linkage at one or more glycosylation sites. Seeds of soybean (*Glycine max* L. Merrill) contain from 0.6% to as much as 6.2% dry weight of triterpenoid saponins depending on the variety, cultivation year, location grown and degree of maturity (Fenwick and Oakenfull, 1981; 1983; Oakenfull, 1981; Shiraiwa *et al.*, 1991a), although typical concentrations appear to be around 1%. The soy saponins have been divided into group A, B and E saponins on the basis of their aglycone structure (Shiraiwa *et al.*, 1991b,c; Kuduo *et al.*, 1992). Group B (and possibly E) saponins appear to exist in the intact plant tissue as conjugates of 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) at the 22 hydroxyl position (Kuduo *et al.*, 1993). These conjugates are relatively labile and are easily degraded during most commercial extraction and processing methods to form the group B and E saponins (Ireland and Dziedzic, 1986; Daveby *et al.*, 1998). Group A saponins appear to be a naturally occurring form. The chemical structures of more than 20 saponins from soybeans and various soy products (many of which are artefacts formed during extraction and analysis) have been determined over the years by a variety of workers and the structures and various naming protocols are summarised in Fig. 1.

Saponins have been reputed to have important biological activities in humans, including hypocholesterolaemic, haemolytic, immunostimulatory and anti-tumourigenic activities (Hostettmann and Marston, 1995), as well as chemoprotective activities (Berhow *et al.*, 2000). Despite a great deal of analytical research over the past 30 years, detailed studies of the biological activities have been hampered owing to the lack of availability of large quantities of purified saponins, as well as the lack of efficient procedures for the detection and quantification of these compounds in foods and food products. A number of acceptable HPLC protocols exist (Shiraiwa *et al.*, 1991a–c; Kuduo *et al.*, 1994; Cui *et al.*, 2000; Mandarino *et al.*, 2000), but the preparation of pure analytical standards is laborious and difficult owing to the large numbers of very similar saponin glycosides. An industrial purification process has been developed to produce kg quantities of highly refined saponins from soy processing co-products. Using this material, we have been able to prepare fractions of highly purified and fully characterised soy saponins suitable for use in biological studies and as analytical standards for quantification.

EXPERIMENTAL

Materials. Whole soybeans were obtained from a local commercial grower. Products produced from the processing of commercially grown soybeans were obtained from Central Soya Company (Fort Wayne, IN, USA), Solbar Hatzor (Ashdod, Israel), and Organic Technolo-

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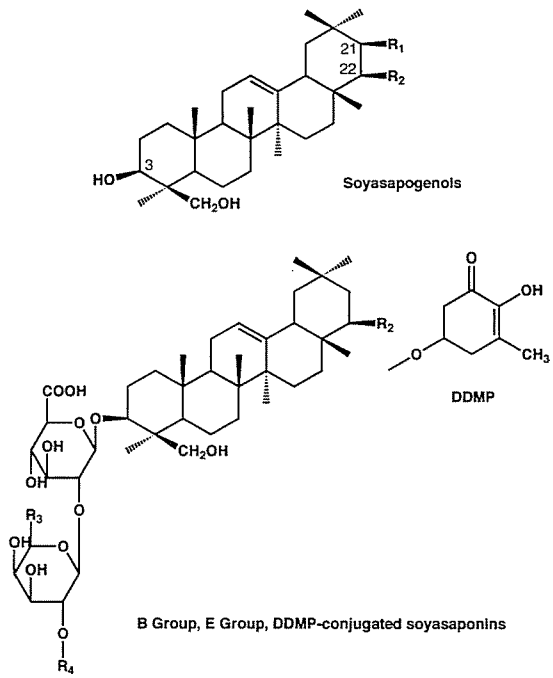

Soyasapogenol A	R_1 = OH	R_2 = OH
Soyasapogenol B	R_1 = H	R_2 = OH
Soyasapogenol E	R_1 = H	R_2 = O

DDMP-conjugated saponins	R_2 = DDMP

Soyasaponins	Group B	Group E	DDMP	R_3	R_4
Soyasaponin	I (Bb)	(Be)	βg	CH_2OH	β-D-Gluc
Soyasaponin	II (Bc)		βa	H	α-L-Rham
Soyasaponin	III (Bb')		γg	CH_2OH	H
Soyasaponin	IV (Bc')		γa	H	H
Soyasaponin	V (Ba)	(Bd)	αg	CH_2OH	α-L-Rham

Figure 1. Structures of soybean saponins. Group B nomenclature is from Kitagawa *et al.* (1988) with the nomenclature of Kuduo *et al.* (1994) in parentheses. The nomenclature of group E and the DDMP-conjugated saponins is from Kuduo *et al.* (1993) and Yoshiki *et al.* (1998), respectively. The nomenclature for the group A saponins (not shown) is more complicated (Shiraiwa *et al.*, 1991c).

gies (Coshockton, OH, USA). All other chemicals and solvents were of the highest degree of purity or spectroscopic grade obtainable.

Isolation of the saponin fraction from whole soybeans. A modified method of Tani *et al.* (1985) was used. Dehulled soybeans were ground to a fine powder and extracted with hexane in a Soxhlet extractor overnight. The dried solid residue was then extracted with methanol in a Soxhlet extractor for 48 h, the methanol extract evaporated, resuspended in 80% aqueous methanol and any remaining solid residue removed by filtration. The supernatant was diluted to 20% methanol by the addition of water, and loaded onto an equilibrated preparative C_{18} reverse-phase column (125 Å; 55–105 μm; Waters, Milford, MA, USA). The column was washed extensively with 70% aqueous methanol to remove the isoflavonoids and the saponins were then eluted with 100% methanol. Finally, the column was cleaned with isopropanol. The resulting eluate was diluted to 10% methanol with water. Under certain conditions (if the concentration of saponins was high enough) a saponin precipitate formed which could be collected by centrifugation. Resuspension and repeated precipitation in this manner resulted in the further separation of the saponins from the isoflavones. In either case, the fractions were reintroduced to a regenerated preparative C_{18} reverse-phase column, washed with 70% aqueous methanol and eluted with 100% methanol. The process was repeated as required to remove any contaminating isoflavones. This process yielded a fraction that was enriched in the group B soyasaponins, which could be used for preparative HPLC as described below.

Isolation of saponins from a refined soy fraction. A sample (2 g) of refined soy saponin fraction (Organic Technologies) containing over 90% (w/w) group B soyasaponins was resuspended in 10 mL dimethylsulphoxide:methanol (1:1), diluted to 50% organic with water and loaded onto a preparative C_{18} column (400 × 160 mm i.d.) equilibrated in 20% aqueous methanol. The column was washed with 50% aqueous methanol and then eluted with an extensive wash of 80% aqueous methanol to yield about 1 g of a fraction (OTC70) containing five soyasaponins (98%).

Sample preparation for HPLC analysis. Weighed samples of the various dried, powdered processing products were suspended in a volume of 80% aqueous methanol and submitted to extensive sonication. Aliquots were filtered prior to HPLC analysis.

Preparative HPLC separation of the soyasaponins. Separation was achieved using a Shimadzu (Kyoto, Japan) preparative HPLC system consisting of two LC-8A preparative pumps, an SCL-10A VP controller, an SPD-M10A PDA, and an SIL-10A auto-injector running under the Class VP software system. The separation was carried out on a C_{18} reverse-phase Inertsil ODS-3 (Metachem Technologies, Torrance, CA, USA) column (250 × 30 mm i.d.; 5 μm). The isocratic mobile phase was 40% aqueous acetonitrile with 0.025% trifluoroacetic acid (TFA) employed at a flow rate of 30 mL/min; 30 mL fractions were collected. The purified soyasaponin fraction (OTC70) was resuspended in 80% aqueous methanol to a concentration of 25 mg/mL and an 1.5 mL aliquot was injected. Five peaks were resolved and the appropriate fractions pooled and evaporated to dryness. The procedure was repeated 15 times to yield approximately 160 mg of soyasaponin I, 44 mg of soyasaponin II, 8 mg of soyasaponin III, 5 mg of soyasaponin IV, and 18 mg of a compound initially identified as soyasaponin V, as confirmed on a molecular weight basis by LC-MS.

LC-MS analysis. Analysis of the various fractions obtained during the process of purification was performed on an HPLC system run isocratically through an electrospray ionisation–mass spectrometer (ESI-MS). The analysis was carried out on a Thermoquest (San Jose, CA, USA) HPLC system and an LCQ-MS system, all running under the Xcalibur computer control system. The HPLC separation was carried out using a C_{18} reverse-phase Inertsil ODS-3 (Metachem Technologies) column (150 × 3 mm i.d.; 3 μm). The isocratic mobile

phase was 80% aqueous methanol with 0.25% acetic acid employed at a flow rate of 0.3 mL/min. The temperature of the inlet capillary was set at 220°C and the MS was auto-tuned using the software by infusion of a sample containing soyasaponin I ($[M + H]^+ = 943$) into the flow stream from the column. Typically 5 μL of filtered samples (100–500 ng/μL) were injected into the system. Full MS were obtained over the range of 200–1500 *m/z*. Saponins were quantified by MS from standard curves prepared from the SIM traces of the purified soyasaponins using the QuanBrowser module in the Xcalibur software package.

HPLC-photodiode array analysis. Quantitative determination by UV absorbance was performed on a Hewlett-Packard (HP; Palo Alto, CA, USA) series 1100 HPLC-PDA system running under the HP Chemstation software. The LC separation was performed on a C_{18} reverse phase Inertsil ODS-3 (Metachem Technologies) column (250 × 4.6 mm i.d.; 5 μm). The isocratic mobile phase was 40% aqueous acetonitrile with 0.025% TFA employed at a flow rate of 1 mL/min and the effluent was monitored at 210 nm. Saponins were quantified from the absorbencies at 210 nm of standard solutions, prepared from purified group B soyasaponins (I, II, III and IV), using the HP Chemstation software package.

Analysis of aglycone (sapogenol). Purified soy saponins (2 g) were hydrolysed with 2 M hydrochloric acid in methanol under refluxing conditions for 48 h. Addition of water to the cooled solution resulted in the formation of a precipitate which was filtered, washed extensively with water to remove residual sugars and acid, resuspended in methanol, and dried in a fume cupboard. A portion of the dried precipitate was then resuspended in methanol, applied to a pre-coated silica gel 60 F_{254} plate (Merck, Darmstadt, Germany) and developed with dichloromethane: methanol (9:1). The plate was sprayed with a saturated solution of potassium dichromate in sulphuric acid and charred by heating to develop reddish-purple spots, indicating the presence of sapogenols. Smaller portions of pure standards were hydrolysed as described above and spotted directly onto the TLC plates.

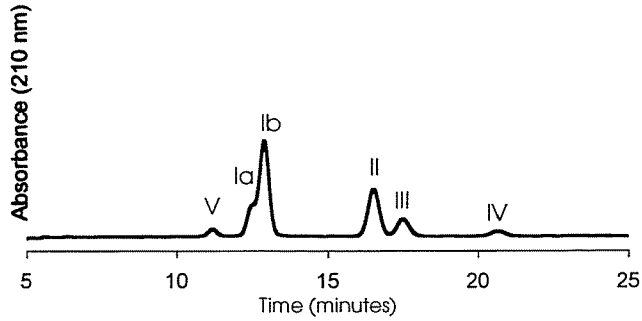

Figure 2. HPLC chromatogram with detection at 210 nm of an analytical run of the purified soy saponin fraction OTC70. (For chromatographic details see the Experimental section.)

NMR analyses. ^{1}H- and ^{13}C-NMR spectra were obtained on a Bruker (Rheinstetten, Germany) Avance 400 MHz spectrophotometer. Samples (5–10 mg) were dissolved in DMSO-*d6*.

RESULTS AND DISCUSSION

Although there have been numerous reports on the analysis of saponin glycosides in soybeans and other legume seeds, many of these procedures are fairly

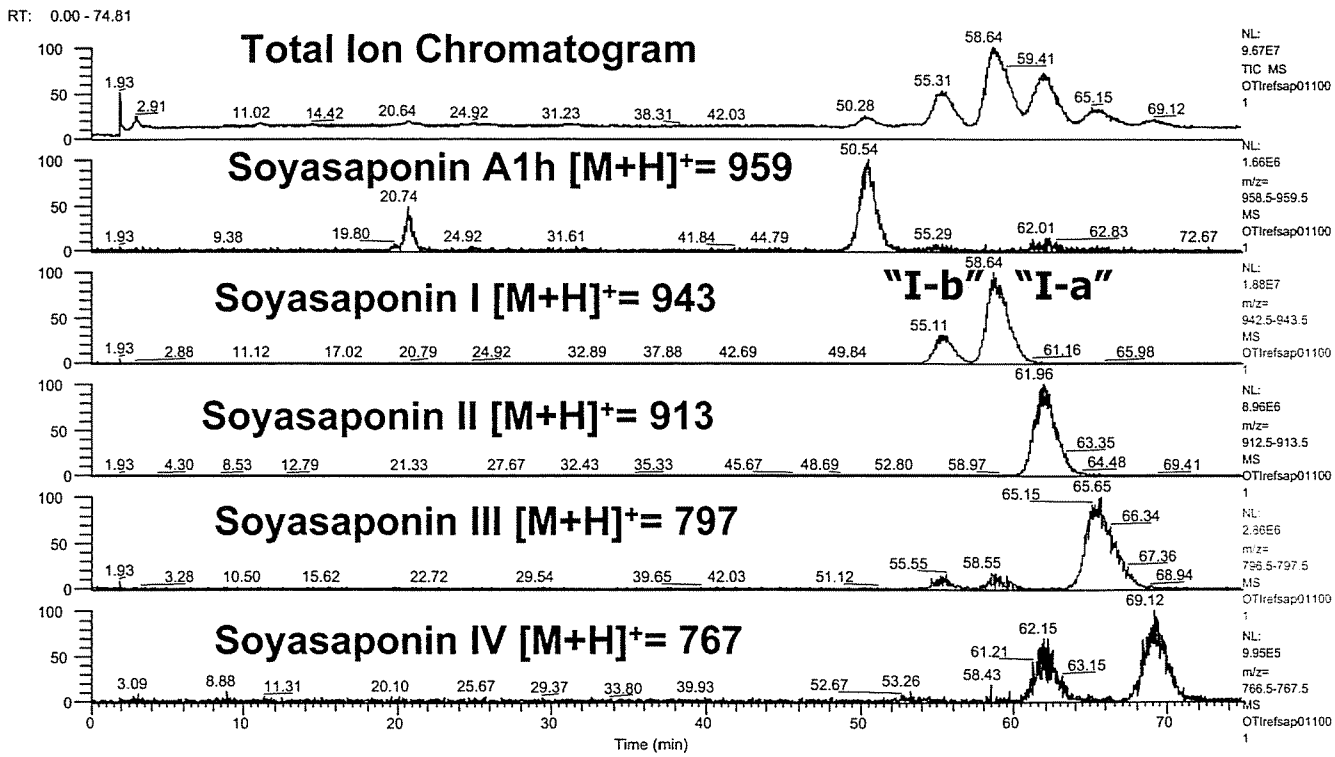

Figure 3. LC-MS traces of an analytical run of the purified soy saponin fraction OTC70. The upper trace is the total ion chromatogram (TIC) whilst the traces below this are SIM traces for *m/z* values of 959, 943, 913, 797 and 767, respectively.

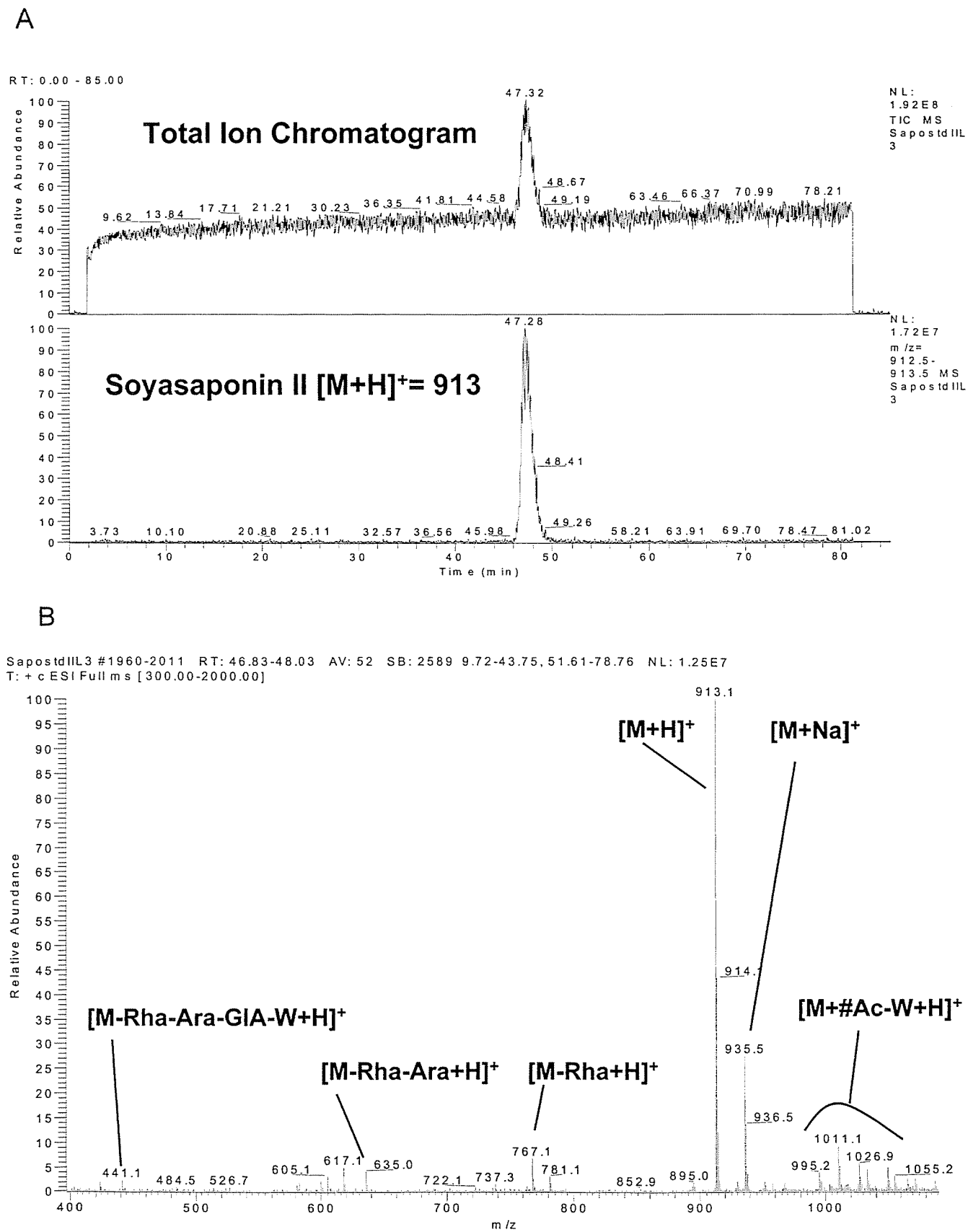

Figure 4. (A) LC-MS traces of an analytical run of pure soyasaponin II. The upper trace is the total ion chromatogram (TIC) whilst the lower trace is the SIM trace for *m/z* value 913. (B) The mass spectra obtained at the TIC peak maximum as shown in the upper trace in (A).

complex. Most sample preparation procedures promote the alteration of the chemical structure of the actual naturally occurring saponins and the result is a bewildering array of 20 or more forms of saponins found in processed soy products. In general, the routine analysis of saponins in soybeans and derived processed products has

Table 1. Analysis of the soyasaponin content of various samples processed from soy

Component	Concentration of soyasaponin (mg/g)[a] in sample[b]												
	IF 1	IF 3	IF 5	IF A	IF B	IF C	IF D	SB 1	SB 5	SB 8	SB 9	SB 11	SB 13
Soyasaponin I	13	43	43	111	89	43	86	—	53	2	2	6	25
Soyasaponin II	—	—	14	32	26	—	2	—	15	tr[c]	1	2	10
Soyasaponin III	1	2	6	9	10	2	43	—	6	tr	tr	1	4
Soyasaponin IV	—	—	1	1	2	—	5	—	1	—	—	tr	1
Soyasaponin A1h	5	13	3	7	5	14	7	—	4	tr	tr	1	2
Total soyasaponins (%)	1.9	5.8	6.7	16.4	13.4	5.8	14.3	0.0	7.9	0.3	0.4	1.0	4.2

	OT-19	OT-50	OT-75	OT-98	OT-RS	OTC70	OTC70 (SIM-MS)[d]	CS 1	CS 2	CS 3	CS 4	CS 5
Soyasaponin I	136	322	479	537	516	557	511	391	37	50	45	33
Soyasaponin II	40	117	209	210	239	256	180	186	5	12	9	3
Soyasaponin III	13	32	100	87	121	124	460	84	3	8	5	2
Soyasaponin IV	4	9	18	20	22	23	18	17	—	tr[c]	tr	—
Soyasaponin A1h	1	18	28	31	28	31	tr	21	—	—	—	4
Total soyasaponins (%)	19.3	49.9	83.4	88.6	92.9	99.1	117.0	69.8	4.5	7	5.9	5.6

[a] Quantitative determinations made using the PDA method unless otherwise indicated.
[b] Key to sample identities: IF, various isoflavone samples; SB, samples from Solbar Hatzor; OT, samples from Organic Technologies; CS, samples from Central Soya.
[c] tr, trace amount detected.
[d] Quantitative determination made using the SIM method.

been hampered by the lack of availability of suitable standards for quantitative determination. Group B saponins may be naturally occurring, but are more likely to be formed from the hydrolysis of the DDMP saponin forms (Daveby *et al.*, 1998), as are the group E saponins.

A proprietary method for the production of a refined saponin product from soy has been developed by Organic Technologies and, using this product, we were able to isolate four group B saponins (I–IV) by preparative HPLC. The identity and elution order of these standards was established by LC-MS based on the characteristic $[M + H]^+$ and $[M + Na]^+$ ions. A fraction of the refined product (OTC70) was separated by preparative reverse-phase C_{18} chromatography and shown to contain only soy saponin glycosides (soyasaponins): from fraction OTC70, five individual soyasaponins were obtained. The HPLC trace for this fraction is shown in Fig. 2, and the LC-MS analysis is shown in Fig. 3. The LC-MS analysis of pure soyasaponin II is shown in Fig. 4. Using the standard materials so derived, a number of processing fractions prepared from soy were evaluated for their group B saponin content; the results are shown in Table 1.

The complete acid hydrolysis of pure soy saponin fraction resulted in at least three spots in the TLC analysis of the resulting sapogenols. The major spot was isolated by preparative TLC (99% pure), and the ^{13}C-NMR data for this fraction were in complete agreement with those reported for soyasapogenol B (Agrawal and Jain, 1992). The ^{1}H-NMR data were in agreement with those predicted for sapogenol B. Further LC-MS analysis of these sapogenols show the presence of two forms of soyasapogenol B and possibly two forms of soyasapogenol A. Hydrolysis of the purified saponin glycoside which was first to elute from the HPLC (initially identified as soyasaponin V) yielded, by TLC, an additional sapogenol spot. It appears that this initially eluting compound is actually a hydrolysis product of soyasaponin A1, yielding a compound (reference soyasaponin A1h) with a molecular weight of 958. Confirmation of this structure is currently being studied in our laboratory.

Fraction OTC70 was analysed quantitatively by HPLC-PDA as well as by LC-MS (Fig. 3) and shown to be composed of 99% soy saponins. The data determined by ion trap MS provides some key structural information in determining the identity of the saponins eluted from the chromatography column (van Setten *et al.*, 2000). The ratios of the five saponins were different when determined by SIM-MS performed by the LC-MS software, than when determined by PDA (Table 2). The levels of mass ions in the generated MS can vary considerably depending on sample preparation, LC conditions, and the degree of ion contamination in the MS itself. This is especially important in the positive mode, resulting in variable numbers and altered relative amounts of mass ions generated as shown in Fig. 4(b). Conditions for the negative ion mode can be optimised to enhance the $[M\text{-}H]^-$ ion, maximising its signal in the MS, but even this signal can vary in day-to-day operation of the MS. In the present study, MS was employed to confirm elution order whilst quantitative determination of the samples was carried out using the absorbance at 210 nm (Fig. 2, Table 1). Samples IF 1, IF 3 and IF 5 derived from whole soybeans (Table 1) contained significant quantities of isoflavones, but these were eluted early under the isocratic conditions of the analytical procedure and did not interfere with the saponin analysis. Other compounds with saponin-like qualities eluted prior to soyasaponin I and interfered with the analysis of soyasaponin A1h.

It was observed that variation in the content of the soy extracts resulted in variation of the elution profiles derived from the preparative C_{18} column. It is important to assess carefully the elution profile of samples prepared from various sources of soy (e.g. from the hypocotyl vs. the endosperm) or from other legumes, for differences in

the relative elution profiles of the isoflavones and the saponins in the bulk separation. As the naturally occurring DMPP-conjugated form of soybean saponins is easily hydrolysed during the course of extraction and processing, it is probably better to drive this hydrolysis to completion and quantify the B group saponins (those most prevalent in processed products derived from soy) rather than try to measure the DMPP-conjugated form for which standards are not available.

Based on the procedure developed by Organic Technologies, it is now possible to produce gramme quantities of a fraction that contains only five soyasaponins at higher than 99% purity. Such material can be used as a standard for the quantification of these compounds. It should now be possible to produce sufficient quantities of purified and characterised soy B group saponins to meet the need for nutritional studies with animals. This will allow for the assessment of this group of food components in the prevention of certain chronic diseases such as cancer and heart disease.

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